The EMBO Journal vol.14 no.1 pp.143–150, 1995

Human RPB5, a subunit shared by eukaryotic nuclear RNA polymerases, binds human hepatitis B virus X protein and may play a role in X transactivation

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Communicated by H.Schaller

The X gene of human hepatitis B virus encodes the polypeptide HBx which transactivates viral and host genes through a variety of *cis*-acting enhancer elements present in RNA polymerases I, II and III promoters. To better understand the mechanism of X transactivation, we cloned cDNAs of proteins that bind HBx. Here we demonstrate that one of these cDNAs is a full-length cDNA of human RPB5, a subunit shared by RNA polymerases. The HBx transactivation domain and the central region of human RPB5 were necessary for the specific binding of the two proteins as shown by: (i) *in vitro* assays using deletion mutants of fusion proteins; (ii) *in vivo* assays which detect associated proteins by co-immunoprecipitation of the non-fused proteins from transfected HepG2 cells. Over-expressed HBx seemed to associate with assembled forms of endogenous human RPB5 in HBx-transfected cells, since the endogenous RPB5 co-immunoprecipitated with HBx. The HBx binding region of human RPB5 by itself stimulated chloramphenicol acetyltransferase activities from several different reporters having X-responsive element(s). Our results support the idea that the interaction of HBx and human RPB5 can facilitate HBx transactivation and that human RPB5 has a domain which can communicate with transcriptional regulators.

Key words: HBV X protein/human RPB5/RNA polymerases/transactivation/X-associated protein

Introduction

Human hepatitis B virus (HBV) belongs to one of the families of small DNA viruses, Hepadnaviridae. HBV has a compact genome 3.2 kb in size. The HBV genome contains the X gene as well as the surface, core and polymerase genes (Tiollais *et al.*, 1985). The product of the HBV X gene, HBx, consists of 154 amino acid residues and the X protein is conserved among mammalian hepadnaviruses (Kodama *et al.*, 1985). The X protein has recently been reported to be essential for establishment of infection (Chen *et al.*, 1993). At a molecular level, HBx transactivates viral and host genes through a wide variety of *cis* elements present in RNA polymerase II promoters, including AP-1, AP-2, ATF, C/EBP, NF-κB sites, SRE (Twu and Schloemer, 1987; Seto *et al.*, 1988; Twu and Robinson, 1989; Faktor and Shaul, 1990; Seto *et al.*, 1990; Luber *et al.*, 1991; Mahe *et al.*, 1991; Lucito and Schneider, 1992; Lucito and Schneider, 1992; Avantaggiati *et al.*, 1993) and one of the RNA polymerase III promoters (Aufiero and Schneider, 1990). Several endogenous genes important for cell proliferation and the inflammatory response are activated by HBx, such as c-*fos*, c-*jun*, ECAM and human IL8 (Mahe *et al.*, 1991; Hu *et al.*, 1992; Avantaggiati *et al.*, 1993; Twu *et al.*, 1993). In this latter context, HBx has been thought to play a positive role in hepatocellular carcinogenesis (Hohne *et al.*, 1990; Kim *et al.*, 1991).

Since HBx has no ability to bind DNA, protein–protein interaction seems to be crucial for HBx transactivation. HBx deletion analyses demonstrated that a transactivation domain was encoded in the latter two thirds of HBx (Arii *et al.*, 1992; Runkel *et al.*, 1993; Murakami, 1994b), while the N-terminal one third of HBx had the ability to repress the transactivation capabilities of HBx. These results indicated that HBx contains at least two functional domains, the transactivation domain and the regulatory domain (Murakami, 1994b). Several mechanisms for X transactivation have been proposed, including HBx binding of transcriptional activators and the utilization of kinase activation pathways, but these mechanisms remain controversial (Takada and Koike, 1990; Hugh *et al.*, 1991; Mahe *et al.*, 1991; Lucito and Schneider, 1992; Avantaggiati *et al.*, 1993; Cross, *et al.*, 1993; Kekule *et al.*, 1993; Murakami *et al.*, 1994a).

One approach to identify the mechanism of HBx transactivation is to identify host proteins which interact specifically with HBx. We screened a λgt11 human cDNA library derived from the hepatoma cell line HepG2 using labeled recombinant HBx as the probe in an *in situ* protein–protein binding reaction (far-Western method) (Kaelin *et al.*, 1992; Murakami *et al.*, 1994b). Here we show that one of the cDNAs we isolated is identical to human RPB5, a common subunit of RNA polymerases I–III (Pati and Weissman, 1989; Woychik *et al.*, 1990; Young, 1991), and that the specific association of HBx and human RPB5 could be detected *in vitro* and *in vivo*. We also provide evidence suggesting that the association of the HBx with human RPB5 mediates the HBx transactivation.

Results

Cloning of cDNAs encoding HBx-associated proteins

A λgt11 cDNA library of a human hepatoma cell line, HepG2, was screened by a far-Western method using a recombinant fusion protein of HBx which was phosphorylated *in vitro* with [γ-^{32}P]ATP (Kaelin *et al.*, 1992; Murakami *et al.*, 1994b). Four positive clones were

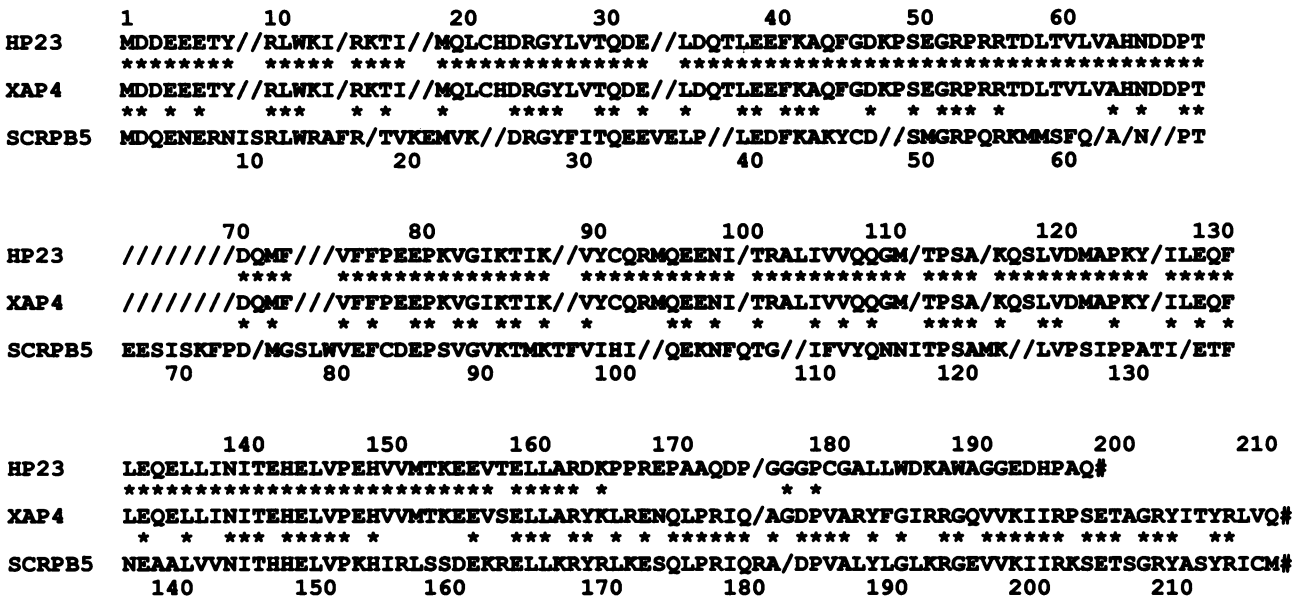

Fig. 1. Alignment of the amino acid sequences of human and yeast RPB5s. Simple alignment of amino acid sequences of XAP4 and HP23 (Pati and Weissman, 1989) and maximum alignment between XAP4 and yeast RPB5 (Woychik *et al.*, 1990) are shown. Numbering of amino acid residues begins at the initiation codon of HP23 (Pati and Weissman, 1989). The nucleotide sequence of the insert of clone 4 was determined as described in Materials and methods. The nucleotide sequence of XAP4 in the coding region is the same as HP23, except for three base substitutions (at 493, 511 and 518 of HP23) and one base deletion (at 521). Because of this deletion, the C-terminal end of XAP4 encodes 210 amino acids and is different from HP23 (197 amino acids). The putative ORF of the XAP4 cDNA fused to the β-galactosidase gene of *E.coli* had an additional 10 amino acids at the junction derived from the linker staff and the 5′-non-translated sequence of HP23 (Pati and Weissman, 1989).

selected from a library containing 8×10^5 plaques. The sequences of three of the clones had no strong homology to any known genes in Genbank. Further characterization of these clones is underway. Clone 4, XAP4, is 1236 bp in length (omitting the 18 bp of the artificial staffs) and the sequence of clone 4 matched the reported sequence of HP23 of human RPB5 (Pati and Weissman, 1989) by 98.1%. Two-thirds of the N-terminal part of the putative HP23 ORF is identical to the putative XAP4 ORF. However, the amino acid sequence of the HP23 ORF (197 amino acids) is different from the XAP4 ORF (210 amino acids) in the C-terminal region by frame-shifting due to a one base deletion. The C-terminal region of the XAP4 ORF has high homology with RPB5 of *Saccharomyces cerevisiae*, one of the common subunits among RNA polymerases I–III, as pointed out by Sentenac and co-workers (Pati and Weissman, 1989; Woychik *et al.*, 1990; Sentenac *et al.*, 1992) (Figure 1).

HBx associated with human RPB5 in vitro

Specific association between HBx and human RPB5 (XAP4) was examined by far-Western blotting using labeled glutathione S-transferase (GST)–HBx or GST–human RPB5 (XAP4) under the same binding conditions employed in the screening of XAP (Figures 2–4). The human RPB5 probe bound specifically to the full-length HBx, HBx-5D3 (21–154) and HBx-5D1 (51–154), all of which harbor the transacting domain, but human RPB5 did not bind to HBx fusion proteins containing the regulatory domain [see HBx-3D5 (1–50) in Figure 2B]. This result indicates that HBx has two different regions for protein–protein interactions, one for self-association in the regulatory domain and the other for human RPB5 binding in the transactivation domain. We recently reported that HBx consisted of two functional domains, the trans-activation domain (HBx-5D1) and the regulatory domain (HBx-3D5). The regulatory domain represses the trans-activation in *trans* and is important in the multimerization of HBx (Murakami *et al.*, 1994b) (Figure 2A).

We next examined whether the human RPB5 binding region of HBx co-localized with the transactivating ability of HBx. The N-terminal delineation of the human RPB5 binding activity coincided well with the transactivating activity of HBx, since HBx-5D12 (57–154), which has reduced transactivating ability, has decreased human RPB5 binding activity and transactivation-negative HBx-5D4 (72–154) barely bound to the human RPB5 probe (Figure 3). In contrast, the C-terminal margin of human RPB5 binding activity may be within the HBx transactivation domain, since a transactivating-negative truncated mutant, HBx-H39 (1–138), exhibited slightly reduced human RPB5 binding activity, and a transactivation-impaired mutant of HBx, HBxm111, which has a substitution of three amino acid residues at 111–113, exhibited similar human RPB5 binding activity to that of the full-length HBx (Figure 3).

Human RPB5 may be present in a dimeric form in RNA polymerases, because it is found in a 2-fold molar amount relative to other polymerase subunits in purified RNA polymerase fractions (Young, 1991). To delineate regions of human RPB5 necessary for HBx binding and for putative self-interaction, deletion mutants of human RPB5 (XAP4) were constructed including d4 (1–46), d13 (47–120), d2 (121–210), d1 (47–210), d3 (1–120) and d5 (1–160) (Figure 4B).

We found that the HBx probe associated with the full-length human RPB5 and also with constructs which harbor the central part of human RPB5, such as RPB5-d13, indicating that HBx bound to human RPB5 through the central region of human RPB5 [RPB5-d13 (47–120)]

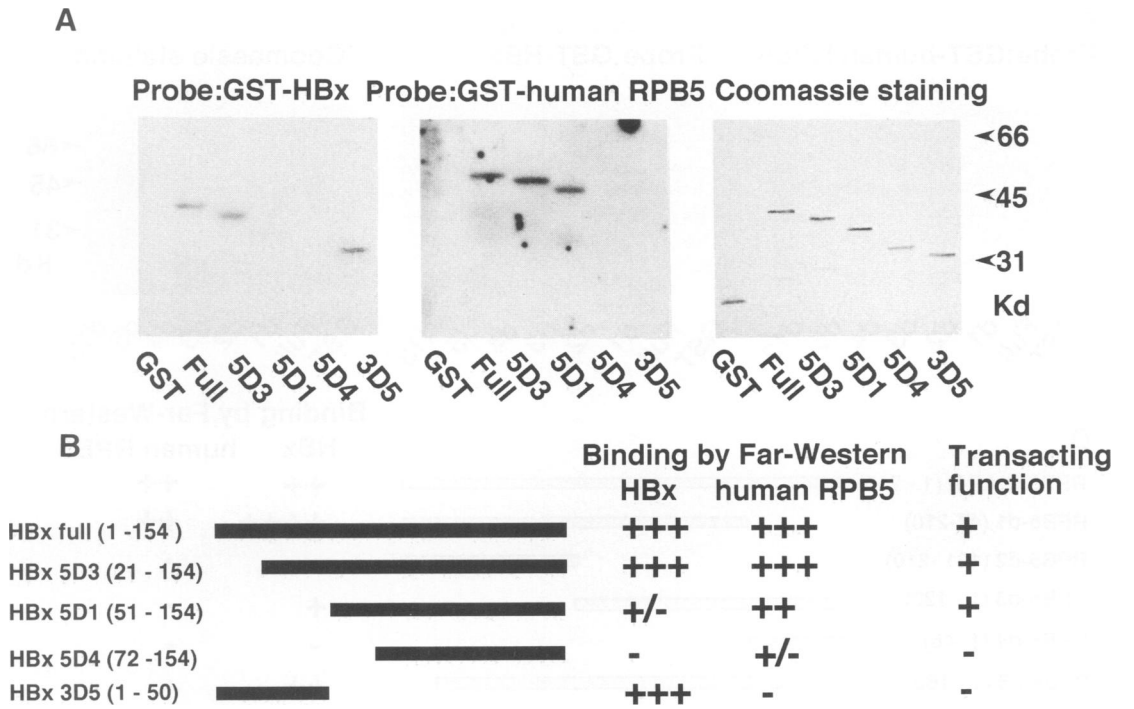

Fig. 2. Specific association of HBx and human RPB5 (XAP4) detected by far-Western blotting. (**A**) Binding activity of the full-length and truncated HBx proteins. The GST fusion proteins of the indicated HBx constructs were expressed in *E.coli*, purified and fractionated on 12.5% SDS−PAGE (Murakami *et al.*, 1994b). Proteins were stained directly with Coomassie Brilliant Blue (the right panel) or transferred to nitrocellulose membranes and subjected to far-Western blotting using ^{32}P-labeled GST−HBx (left panel) or ^{32}P-labeled GST−human RPB5 (XAP4) (middle panel) as the probe. Far-Western blotting was carried out as described in Materials and methods. Exposure time with the HBx probe and the human RPB5 probe was 5 h. (**B**) Delineation of binding activities of HBx. HBx-5D1, -5D3, -5D4 and -3D5 encode an initiation codon followed by amino acids 51–154, 21–154, 72–154 and 1–50 of HBx respectively (Murakami *et al.*, 1994b). Results of transactivating ability of HBx proteins are shown in Figures 3B and 6A (Murakami *et al.*, 1994b).

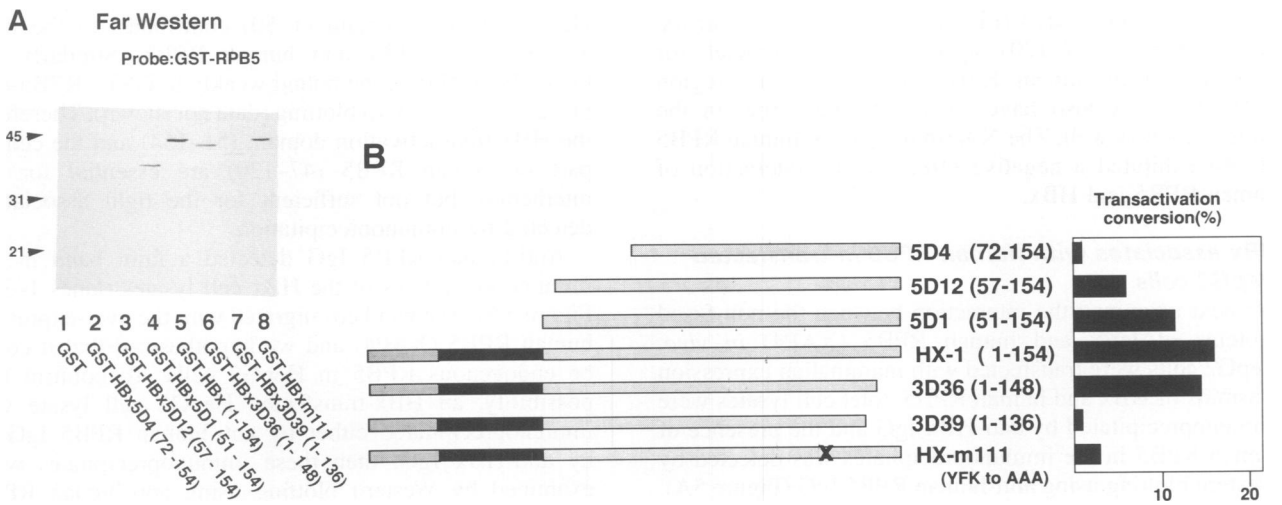

Fig. 3. Delineation of human RPB5 binding ability of HBx. (**A**) Far-Western blotting of GST−HBx fusion mutants was carried out with the ^{32}P-labeled GST−human RPB5 probe as described in Figure 2. (**B**) Deleted or mutated HBx constructs are schematically shown in the left panel. The shaded region is the serine/proline-rich region in the regulatory domain that is essential for self-interaction. Construction of HBx deletion mutants has been described previously (Murakami *et al.*, 1994b). Xm111, having three amino acid substitutions (YFK→AAA) at 111–113 of HBx, was constructed as described in Materials and methods. Transactivation ability of HBxs is shown in the right panel. HepG2 cells were transfected with mammalian expression plasmids of deleted or mutated versions of a HBx construct (1 μg), pSG5UTPL (4 μg) and a CAT reporter, pHECx2CAT (5 μg). Total cell lysates were prepared 48 h after transfection and subjected to CAT analysis (Murakami *et al.*, 1990, 1994a). The CAT reporter pHECx2CAT has a dimerized 23 bp in the center of the 33 bp of the HBV Enh1 core. This region harbors the X-responsive *cis* element(s) and is highly conserved among mammalian hepadnavirus genomes (Murakami *et al.*, 1990). Relative CAT activities (% acetylation) were measured as described in Materials and methods (Mahe *et al.*, 1991).

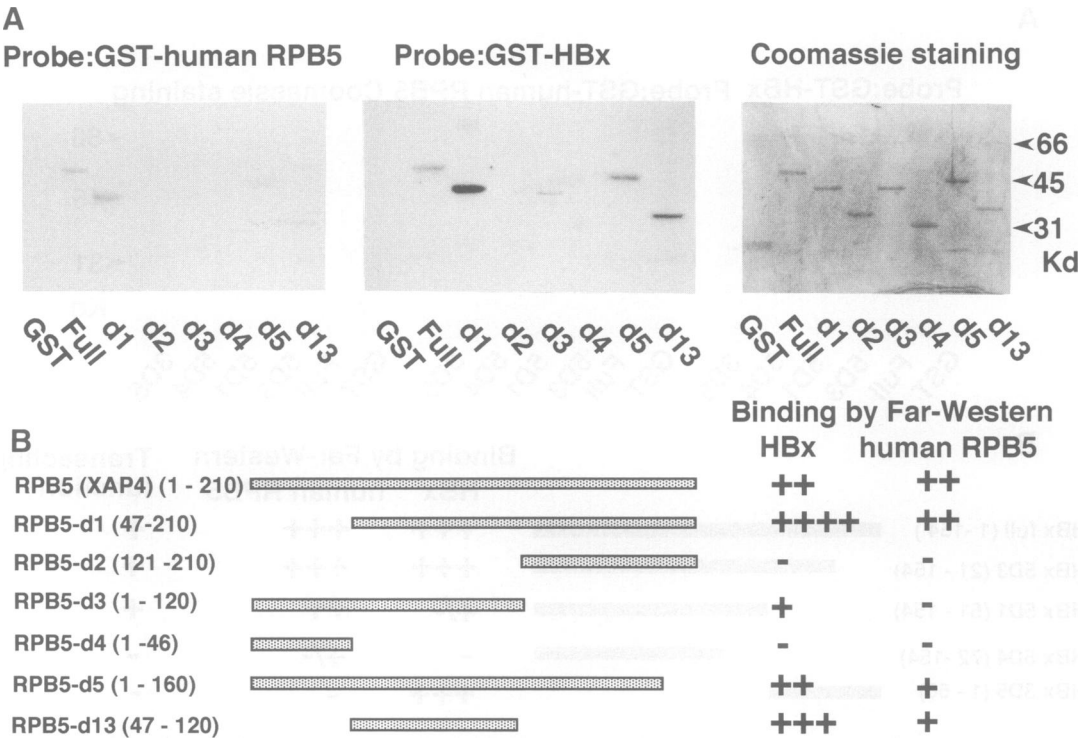

Fig. 4. Binding activity of human RPB5 (XAP4) *in vitro*. (**A**) Far-Western blotting of GST-fused forms of full-length and truncated human RPB5 was carried out with ^{32}P-labeled GST−HBx or GST−human RPB5 (XAP4) as the probe. GST−RPB5-d1 and -d13 have an additional methionine followed by amino acids 47–210 and 47–120 of human RPB5 (XAP4) respectively and GST−RPB5-d2, -d3, -d4 and -d5 have 121–210, 1–120, 1–46 and 1–161 of human RPB5 (XAP4) respectively. Bacterial fusion proteins were prepared and then fractionated on 12.5% SDS−PAGE. Proteins were stained directly with Coomassie Brilliant Blue (the right panel) or transferred to nitrocellulose membranes and subjected to far-Western blotting using ^{32}P-labeled GST−HBx (middle panel) or ^{32}P-labeled GST−human RPB5 (XAP4) (left panel) as the probe. Far-Western blotting was carried out as described in Materials and methods. Exposure times with the HBx probe and the human RPB5 probe were 4 and 10 h respectively. (**B**) Delineation of binding activities of human RPB5 is schematically summarized.

(Figure 4A). The human RPB5 probe interacted with human RPB5-d1 and the full-length human RPB5 and weakly with RPB5-d13 (Figure 4A), suggesting that the central region (47–120) appeared to be crucial for dimerization of human RPB5. The C-terminal region (121–160) may also have some subsidiary role in the dimerization as well. The N-terminal part of human RPB5 (1–46) exhibited a negative effect on the interaction of human RPB5 and HBx.

HBx associates with human RPB5 in transfected HepG2 cells

We next examined the interaction between the non-fused proteins of HBx and human RPB5 (XAP4) *in vivo*. HepG2 cells were transfected with mammalian expression plasmids of HBx and human RPB5, total cell lysates were immunoprecipitated by anti-HBx IgG and the presence of human RPB5 in the immunoprecipitates was detected by Western blotting using anti-human RPB5 IgG (Figure 5A). Anti-HBx IgG immunoprecipitated the full-length HBx complexed with the full-length human RPB5, RPB5-d1 (47–210) and RPB5-d13 (47–120), but did not immunoprecipitate RPB5-d4 (1–46) (data not shown) or RPB5-d2 (121–210) (Figure 5A). Anti-human RPB5 IgG immunoprecipitated the full-length HBx in cell lysates of HepG2 cells co-transfected with mammalian expression plasmids of HBx and human RPB5 (data not shown). Anti-HBx IgG immunoprecipitated HBx-5D1 (51–154) complexed with the full-length human RPB5 and RPB5-d1, but did not precipitate RPB5-d13 (Figure 5A), suggesting that the HBx regulatory domain (1–50) contributes to the tight association of HBx and human RPB5. Similarly the GST−HBx-5D1 probe bound weakly to GST−RPB5-d13 protein in far-Western blotting (data not shown). Therefore, the HBx transactivation domain (51–154) and the central part of human RPB5 (47–120) are essential for the interaction, but not sufficient for the tight association detected by immunoprecipitation.

Anti-human RPB5 IgG detected a faint band in the immunoprecipitates of the HBx cell lysates (lanes 1–3 in Figure 5A). The band co-migrated with the over-expressed human RPB5 (XAP4) and we hypothesized that it could be endogenous RPB5 in HepG2 cells. To confirm this possibility, an HBx-transfected HepG2 cell lysate was immunoprecipitated either by anti-human RPB5 IgG or by anti-HBx IgG, then these immunoprecipitates were examined by Western blotting using anti-human RPB5 IgG (Figure 5B). Approximately 5 ng human RPB5 were detected in the immunoprecipitates with anti-human RPB5 IgG using 450 μg of the HepG2 lysate, while ~0.5 ng human RPB5 were detected in the immunoprecipitates with anti-HBx IgG. These results indicate that the endogenous human RPB5 was associated with HBx in the transfected cells.

We took another approach to examine the interaction of endogenous human RPB5 and overexpressed HBx

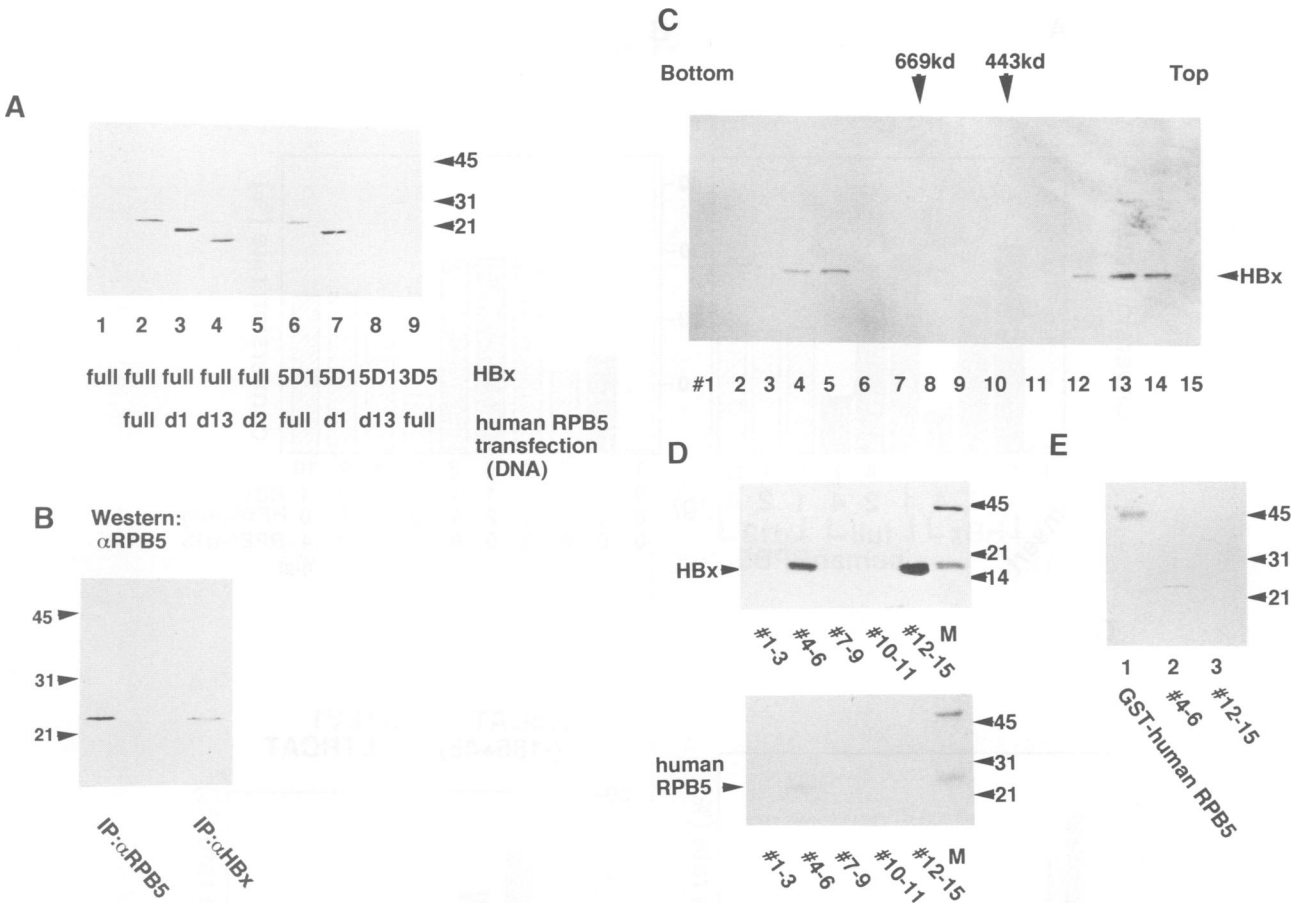

Fig. 5. Immunological detection of association of human RPB5 (XAP4) and HBx. (**A**) HepG2 cells were transfected with mammalian expression vectors of human RPB5 (XAP4) (5 μg) and HBx (5 μg) in addition to a control vector, pSG5UTPL (10 μg). Total lysate proteins were prepared and immunoprecipitated with anti-HBx IgG as described in Materials and methods. The precipitates were fractionated on 12.5% SDS−PAGE and subjected to Western blotting using anti-human RPB5 IgG. (**B**) HepG2 cells were transfected with pSGHX-1 (5 μg) and the control vector (15 μg). Total cell lysates (~450 μg) were reacted with anti-human RPB5 (left lane) or anti-HBx IgG (right lane). The immunoprecipitates were solubilized, fractionated on 12.5% SDS−PAGE, transferred to a nitrocellulose membrane and subjected to Western blotting using anti-human RPB5 IgG. (**C**) Distribution of over-expressed HBx in a glycerol gradient. Total cell lysate was prepared from HBx-transfected HepG2 cells and subjected to 10–35% glycerol gradient centrifugation as described in Materials and methods. After fractionation from the bottom, one-third of each sample was separated on 15% SDS−PAGE, transferred to a nitrocellulose membrane and subjected to Western blotting using anti-HBx IgG. Relative positions of external markers, thyroglobulin and apoferritin (669 and 443 kDa respectively), are indicated. (**D**) Distribution of HBx and the endogenous human RPB5 in combined fractions from a glycerol gradient. One-third of pooled fractions was precipitated with 10% trichloroacetic acid, washed with cold acetone and solubilized in 30 μl. Fifteen microliters of the solubilized sample were fractionated on 15% SDS−PAGE and subjected to Western blotting using anti-HBx IgG (upper panel) and anti-human RPB5 (lower panel) respectively. The two right-most lanes are GST−HBx protein (~2 ng) and thrombin-digested GST−HBx (~2 ng fused protein) respectively. (**E**) The over-expressed HBx recovered in the fast sedimenting fractions was associated with endogenous human RPB5. The remaining two-thirds of the pooled fast sedimenenting fractions (4–6) and the top fractions (12–15) were subjected to immunoprecipitation by anti-HBx IgG. The precipitates were solubilized, fractionated on 15% SDS−PAGE and subjected to Western blotting with anti-human RPB5 IgG. Lanes 1–3 are purified GST−human RPB5 (XAP4) protein (5 ng) and fractions 4–6 and 12–15 respectively.

in vivo. An HBx-transfected HepG2 cell lysate was prepared in the presence of high salt (0.4 M NaCl and 50 mM KCl) and a non-ionic detergent (9 mM CHAPS) and subjected to glycerol gradient centrifugation. Approximately 10–20% of HBx was recovered in the fast sedimenting fractions, indicating that HBX was bound up in a large complex, since it sedimented faster than the 669 kDa marker (thyroglobin) (Figure 5C). The endogenous RPB5 was exclusively detected in the fast sedimenting fractions (Figure 5D). Since the endogenous human RPB5 in the fast sedimented fraction was detected by immunoprecipitation with anti-HBx IgG, this indicated that HBx was complexed with the endogenous human RPB5 in the fast sedimenting fractions (Figure 5E). These two lines of evidence strongly suggest that HBx is associated with the endogenous human RPB5 in large complexes in HepG2 cells.

Transactivating ability of the HBx binding region of human RPB5

If the association of HBx and human RPB5 has biological relevance for HBx transactivation, over-expression of either human RPB5 or the HBx binding region of human RPB5 might affect the enhancer activity of X-responsive *cis* elements. Mammalian expression plasmids of HBx and human RPB5 were introduced into HepG2 cells together with a chloramphenicol acetyltransferase (CAT) reporter that harbors an X-responsive element(s) and CAT activity was measured in lysates of transiently transfected cells. The full-length human RPB5 (XAP4), as well as

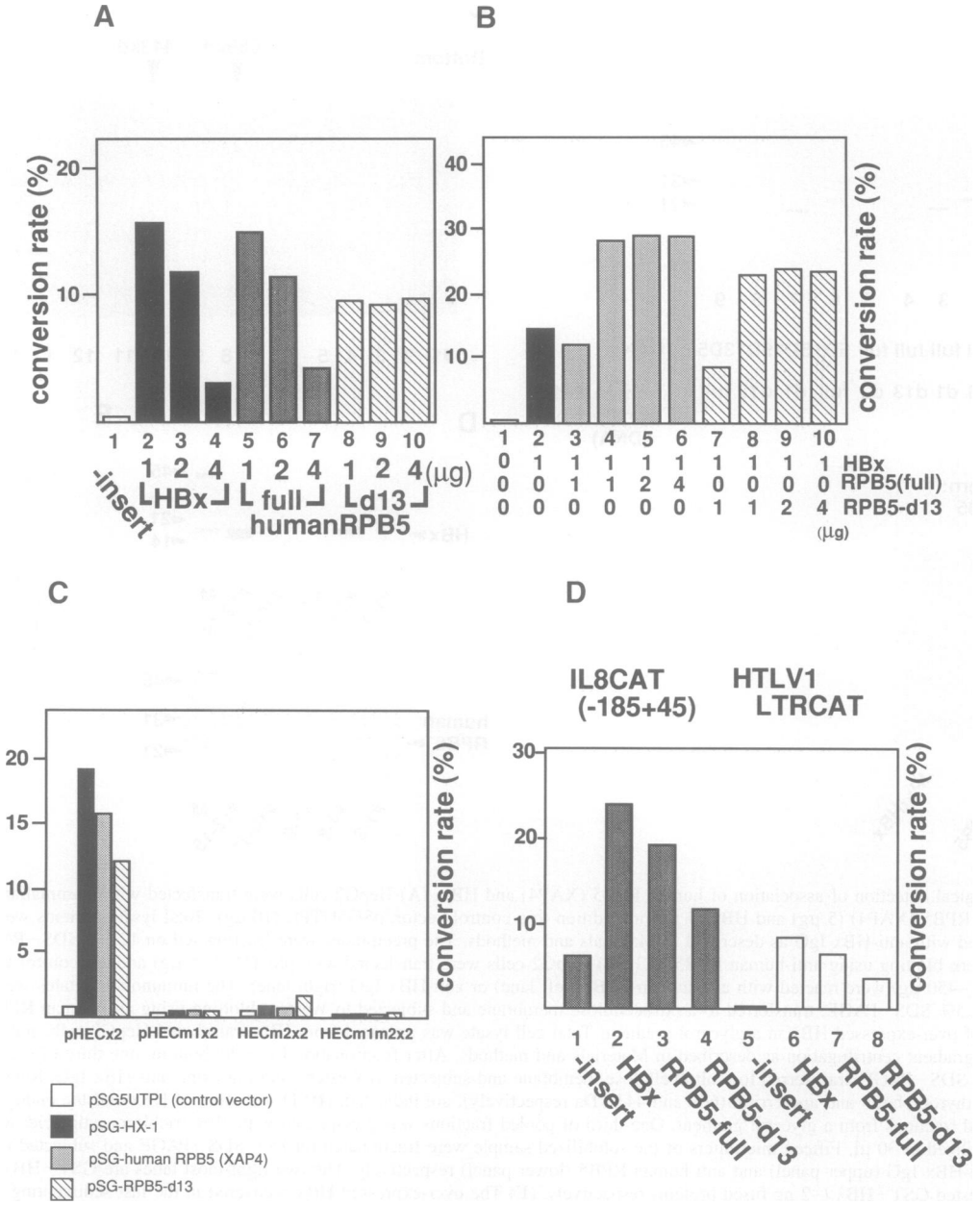

Fig. 6. Human RPB5 stimulates CAT activity in reporters which have an HBx-responsive element(s). HepG2 cells were transfected with a CAT reporter (5 μg) and mammalian expression plasmids for human RPB5 or HBx. Cell lysates were prepared and analyzed for CAT activity as described in Figure 3B. (**A**) Transactivating ability of human RPB5 and HBx (Murakami *et al.*, 1990, 1994b). HepG2 cells were transfected with 5 μg pHECx2CAT and varying amounts of pSG-HX-1 (lanes 2–4), pSG-human RPB5 (XAP4) (lanes 5–7) or pSG-RPB5-d13 (lanes 8–10) respectively. The amount of DNA added per transfection was adjusted to 10 μg with the control vector, pSG5UTPL. (**B**) Additive effect of transactivating functions of HBx and human RPB5 (XAP4). HepG2 cells were transfected with a constant amount of pHECx2CAT (5 μg) and pSG-HX-1 (1 μg) and varying amounts of pSG-human RPB5 (XAP4) or pSG-RPB5-d13 as described in (A). (**C**) Human RPB5 (XAP4) and the HBx binding region of human RPB5 (RPB5-d13) require X-responsive *cis* elements. Transactivator indicator (1 μg), pSG5UTPL (4 μg) and the CAT reporter indicator (5 μg) were added to the cells. HECm1x2CAT and HECm2x2CAT harbor a two base substitution in the cFAP-1 site and a two base substitution in the C stretch in the 23 bp sequence of the Enh1 core. HECm1m2x2CAT has both of these two base substitutions (Murakami *et al.*, 1994a). (**D**) Human RPB5 (XAP4) and the HBx binding region of human RPB5 (RPB5-d13) transactivated CAT reporters having different X-responsive elements. IL8 (−185) CAT (lanes 1–4) and HTLV1LTRCAT (lanes 5–8) contain −185 to +44 bp of the human interleukin 8 gene and the triple 21 bp repeat of the enhancer of human T cell leukemia virus 1 respectively.

the HBx binding region of human RPB5, RPB5-d13 (47–120), transactivated pHECx2CAT, which has a dimeric 23 bp sequence in the center of the HBV enhancer 1 (Enh1) core (Figure 6A). Human RPB5 and RPB5-d13 can also transactivate other CAT reporters containing a different X-responsive *cis* element(s), including the IL8 regulatory region (Mahe *et al.*, 1991), pSV2CAT (data not shown) and HTLV1 LTR (Nakamura *et al.*, 1989) (Figure 6D). HBx and human RPB5 acted in an additive fashion in CAT assays and the inhibitory effect of human RPB5 was not observed when HBx was present (Figure 6B). The HBx binding region (RPB5-d13) and the HBx transactivation domain (HBx-5D1) also produced additive effects when tested in this assay. (data not shown).

The contribution of the two *cis* elements, the cFAP1 binding site and a stretch of C residues, in the HBV Enh1 core has been studied using CAT reporters having mutations in either or both of these *cis* elements (Murakami *et al.*, 1994a). Transactivation of human RPB5 or RPB5-d13 required both *cis* elements in a manner similar to that for HBx transactivation, as reported previously (Murakami *et al.*, 1994a) (Figure 6C). Therefore, the full-length human RPB5 or the central region of human RPB5 requires X-responsive *cis* elements for transactivation.

Discussion

In order to further define the role that HBx plays in viral and cell proliferation and oncogenesis, we took the approach of cDNA cloning to identify host proteins which associate with HBx and might affect the ability of HBx to transactivate. We identified human RPB5 as one of the proteins which can associate with HBx and demonstrated the specific association of HBx and human RPB5 *in vitro* by far-Western blotting experiments. The association of HBx and human RPB5 was also detected in transfected HepG2 cells by immunoprecipitation. The transactivation domain of HBx and the central region of human RPB5 (47–120) are essential for the specific interaction *in vitro* and *in vivo*. The HBx binding region of human RPB5 by itself transactivated constructs containing X-responsive elements and acted additively with HBx in transactivation. Taken together, our results support the idea that HBx associates with human RPB5 and stimulates transcription through association of HBx and human RPB5. The association of HBx and human RPB5 may stimulate some sets of enhancers and promoters selectively, since human RPB5 did not transactivate CAT reporters lacking the X-responsive elements.

The N-terminal delineation of the human RPB5 binding region of HBx did not coincide with the N-terminal delineation of the transactivation domain, but the C-terminal mapping of the human RPB5 binding region was within the transactivation domain. Our data seem to be consistent with the results of others which demonstrated two separable regions in the transactivating domain (Runkel *et al.*, 1993). Another factor(s) may interact with the transactivation domain through the C-terminal part of HBx. The identification of host protein(s) which associate with HBx through the C-terminal part of the transactivating domain may elucidate the molecular mechanism of HBx transactivation.

Human RPB5 has been identified as one of the common subunits in RNA polymerases I–III, although its function has not been addressed (Pati and Weissman, 1989; Woychik *et al.*, 1990, Young, 1991). Our results on the interaction of human RPB5 is the first physical evidence for the self-association of RPB5. This association had been expected from evidence that there are two molar equivalents of human RPB5 in RNA polymerases (Young, 1991). We estimated the number of human RPB5 molecules to be around $5–6\times10^4$ molecules/HepG2 cell, since endogenous human RPB5 could not be directly detected by Western blotting (less than 0.5 ng in 30 μg HepG2 lysate protein; data not shown) and ~5 ng human RPB5 could be detected in 450 μg HepG2 protein (Figure 6A and C). If human RPB5 is present at two molar equivalents in RNA polymerases, the total number of RNA polymerases is around $2–3\times10^4$ molecules/HepG2 cell.

Over-expressed HBx was recovered in fast sedimenting glycerol gradient fractions (Koleske and Young, 1994) and was associated with endogenous human RPB5. This result strongly suggests that HBx is associated with endogenous human RPB5 in assembled forms. Since HBx interacts with the central region of human RPB5 and might modulate the transcription process through this interaction, human RPB5 could be a subunit of RNA polymerases which has a surface for the binding of transcriptional factors. Such interaction between transcriptional regulators and a subunit of RNA polymerase has previously been described for the C-terminal region of the α subunit of the prokaryotic RNA polymerase core complex (Ishihama, 1992; Ross *et al.*, 1993).

Materials and methods

Plasmid constructions

The plasmid pSG5UTPL was a mammalian expression vector derived from pSG5 (Stratagene) (Mahe *et al.*, 1991; Murakami *et al.*, 1994a,b). pGENK1 was used as the *Escherichia coli* expression vector for fused GST. These proteins have a phosphorylation site for cAMP-dependent protein kinase (Kaelin *et al.*, 1992; Murakami *et al.*, 1994b). The description of the full-length and truncated HBx expression plasmids has been reported previously (Mahe *et al.*, 1991; Murakami *et al.*, 1994b). Xm111 was constructed by splicing polymerase chain reaction (PCR) (Ho *et al.*, 1989) using oligonucleotides GAGGCAGCCGCAGCAGACTGTTTTAAGGACTGG and TGTCAGTGCTGCGGCTGCCTCAAGGTCGGTCGT, which have three base substitutions resulting in three amino acid substitutions (YFK→AAA) at positions 111–113 of HBx. The full-length coding region of human RPB5 (XAP4) (1–210) was prepared by a PCR cloning method in which a plasmid derived from pBluescript II SK$^-$ having the insert of clone 4 was used as the template. A pair of oligonucleotides, one generating an artificial *Eco*RI site and an initiation codon at the 5′ end of XAP4 and another at the 3′ end which generated a *Bgl*II site, were used for PCR (Murakami, 1994b). The PCR product was digested with *Eco*RI and *Bgl*II and inserted into the *Eco*RI and *Bam*HI sites of pSG5UPTLP and pGENK1. The resulting plasmid, pSG-human RPB5 (XAP4), was isolated and used as the template to construct truncated versions of human RPB5 by PCR cloning. RPB5-d1 and -d13 encode an initiation codon followed by amino acids 47–210 and 47–120 of human RPB5 (XAP4) respectively and RPB5-d2, -d3, -d4 and -d5 encode 121–210, 1–120, 1–46 and 1–161 of human RPB5 (XAP4) respectively. The truncated fragments of human RPB5 were inserted into the *Eco*RI and the *Bgl*II sites of pSG5UTPL. All human RPB5 constructs used in this paper were sequenced using a Taq sequencing primer kit and DNA sequencer 370A (Applied Biosystems).

Cloning of cDNAs encoding X-associated proteins

Approximately 50% (8×10^5 plaques) of a λgt11 cDNA library of HepG2 (HL1105b; Clonetech Laboratories Inc.) was screened. The screening method was essentially as previously reported (Murakami *et al.*, 1994b). In short, the nitrocellulose filters were finally incubated in modified GBT buffer (10% glycerol, 50 mM HEPES–NaOH, pH 7.5, 150 mM KCl, 7.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 1% Triton X-100) and subjected to far-Western blotting. Purified fused HBx was phosphorylated *in vitro* by [γ-^{32}P]ATP using the catalytic subunit of cAMP-dependent protein kinase (Sigma). Protein–protein binding reactions (far-Western) were performed with the labeled probe (100 ng/ml protein, 4×10^6 c.p.m./μg protein) in modified GBT buffer supplemented with 1% BSA, 2 mM unlabeled ATP and the sonicated supernatant of *E.coli* JM109 transformed with pGENK1 containing GST protein at a final concentration of 1 mg/ml (Murakami *et al.*, 1994b). Filters were rotated in the binding mixture for 1 h at room temperature, washed four times with the modified GBT buffer and exposed to X-ray films (XAR Omat, Kodak) for 12 h. At the second screening, several pairs of positive and closely juxtaposed negative plaques on a plate were examined for their inserts by PCR using λgt11 forward and reverse primers. After the third screening, four positive clones, 1–4, were selected. PCR products of these clones were sequenced by a dideoxy sequencing reaction with

Taq polymerase (AmpliTaq; Cetus) and analyzed by ABI 370A (Applied Bio Industry). The *Eco*RI frgament of clone 4 was subcloned into pBluescript II SK$^-$ (Stratagene) and several clones were isolated.

The *Eco*RI fragment was further digested with *Sal*I (386 nt), *Bam*HI (546 nt) or *Pst*I (715 nt), separated and subcloned into pBluescript II SK$^-$.

Independent clones of these constructs were sequenced with Taq polymerase using dye−primer sequencing kits and analyzed by ABI 370A (Applied Bio Industry). Two 20mer primers, the 5′ one beginning at position 805 and the 3′ one at position 880, were synthesized and used for sequencing using the dye−terminater kit (Applied Bio Industry). Sequencing of the coding region was carried out using at least four independent clones and the non-coding region was sequenced using two independent clones. The accession number of the whole nucleotide sequence of the insert of clone 4 in the DNA data bank of Japan (DDBJ) is D38251.

Transfection and CAT assay

Transient transfections and CAT assays were carried out as described previously (Chen and Okayama, 1987; Mahe *et al.*, 1991). A human hepatoma cell line, HepG2, in 60 mm cell culture dishes was used for transfections. Total cell lysates were prepared from cells harvested 48 h after transfection. The CAT assay reactions were carried out for 60 min at 37°C using ~20 μg protein from the transfected lysates (Murakami *et al.*, 1990; Mahe *et al.*, 1991). The fractionated TLC plates were exposed to imaging plates and CAT activities were measured as the percentage of conversion to acetylated forms of [^{14}C]chloramphenicol (% acetylation) (Amersham) using a bioimage analyzer (BA100; Fuji). Transfection and CAT assays were performed at least three times with each combination of transactivator and CAT reporter construct. Representative data are shown.

Western blotting and immunoprecipitation

HepG2 cells were transfected under the same conditions as for CAT assay analyses except that cells were grown in 100 mm cell culture dishes (Murakami *et al.*, 1994b). Total cell lysates in LAC buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 0.4 M NaCl, 10 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM EDTA, 9 mM CHAPS, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and leupeptin) were fractionated by SDS−PAGE. Anti-human RPB5 IgG was isolated from the serum of rabbits immunized with purified recombinant GST−human RPB5 fusion protein containing amino acids 1–197 of XAP4. The antibodies were purified by successive affinity chromatographies using protein A and GST resins. The filters were sequentially treated with anti-human RPB5 IgG, protein A-conjugated horseradish peroxidase (1/5000 dilution) and finally lumino and peroxidase for chemiluminescence, according to the manufacturer's instructions (Amersham).

Total cell lysates of transfected HepG2 cells (~450 μg/100 μl) were reacted with 15 μl of the first IgG in a total volume of 500 μl of GBT buffer. After rocking for 1 h at 4°C, 20 μl of preswollen protein A−Sepharose beads (Pharmacia LKB) were added. Samples were rocked for another 1 h at 4°C, washed three times with washing buffer (10 mM Tris−HCl, pH 7.4, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA) and finally eluted with elution buffer containing 10 mM Tris−HCl, pH 7.4, 2% SDS, 0.3 M NaCl, 1 mM EDTA. The eluted proteins were separated and subjected to Western blotting using the second anti-IgG.

Fractionation of total cell lysates was carried out by glycerol gradient centrifugation. HBx-transfected HepG2 cells (12 plates of 100 mm cell culture dishes) were collected and washed with phosphate-buffered saline, then lyzed with 1 ml of LACII buffer (the same as LAC buffer except omitting glycerol) at 4°C. The lysate was spun in a Microfuge for 5 min to remove debris and then layered onto a Beckman SW40 Ti linear gradient of LACII containing 11 ml of 10–35% glycerol (vol/vol) on 1 ml of a 65% glycerol cushion. Gradients were spun at 18 000 r.p.m. for 20 h at 4°C. Thyroglobulin and apoferritin (669 and 443 kDa respectively; Sigma) were used as external molecular markers. Approximately 15 tubes of 0.75 ml samples were collected and one-third of each sample was fractionated on a 15% acrylamide gel by SDS−PAGE and subjected to Western blotting using anti-HBx IgG. Pooled samples were prepared by pooling three subjacent tubes. A one-third volume of each pooled sample was precipitated with 10% trichloroacetic acid, washed with cold acetone and separated by SDS−PAGE. The distributions of endogenous human RPB5 or HBx were examined by immunoprecipitation. Pooled samples of fast sedimenting fractions (samples 4–6) and slow sedimenting fractions (samples 12–14) were subjected to immunoprecipitation with anti-HBx IgG and the eluated sample was fractionated by SDS−PAGE and subjected to Western blotting with anti-RPB5 IgG.

Acknowledgements

We thank K.Matsushima, M.Fujii, M.Seiki (Cancer Research Institute, Kanazawa University) and A.Ishihama (National Institute of Genetics) for their critical discussions, Janet Embretson (Viromed Laboratories Inc., Minneapolis, MN) for improving the manuscript and N.Hattori (Metropolitan Hospital, Tokyo), K.Kobayashi and S.Kaneko (1st Internal Medicine, Kanazawa University) for encouraging support. We also thank C.Matsushima and K.Terada and T.Nishikawa for sequencing and their technical assistance respectively.

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Received on July 4, 1994; revised on September 27, 1994